

FORM PTO-1390 (Modified)
(REV 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES

213701US0PCT

DESIGNATED/ELECTED OFFICE (DO/EO/US)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

CONCERNING A FILING UNDER 35 U.S.C. 371

09/936179

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/JP00/01478

10 March 2000

10 March 1999

TITLE OF INVENTION

METHOD FOR SCREENING BIOMOLECULE ACTIVITY REGULATOR

APPLICANT(S) FOR DO/EO/US

OKAMOTO Satoru et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☒ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Notice for Consideration of Documents Cited in International Search Report/Notice of Priority
PCT/IB/304/Drawings (4 Sheets)/PCT/IB/308/Sequence Listing (6 sheets) Enclosures

U.S. APPLICATION NO. (IF KNOWN), SEE 37 CFR

09/936179

INTERNATIONAL APPLICATION NO.

PCT/JP00/01478

ATTORNEY'S DOCKET NUMBER

213701US0PCT

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **\$1000.00**
- ☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **\$860.00**
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **\$710.00**
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **\$690.00**
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **\$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =**CALCULATIONS PTO USE ONLY****\$860.00**Surcharge of **\$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).**\$0.00**

| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE |
|--------------------|--------------|--------------|-----------|
| Total claims | 6 - 20 = | 0 | x \$18.00 |
| Independent claims | 1 - 3 = | 0 | x \$80.00 |

\$0.00**\$0.00**Multiple Dependent Claims (check if applicable). ☐**\$0.00****TOTAL OF ABOVE CALCULATIONS =****\$860.00**☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.**\$0.00****SUBTOTAL =****\$860.00**Processing fee of **\$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).**\$0.00****TOTAL NATIONAL FEE =****\$860.00**Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). ☐**\$0.00****TOTAL FEES ENCLOSED =****\$860.00**Amount to be:
refunded

\$

charged

\$

- a. ☒ A check in the amount of **\$860.00** to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **15-0030**. A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

**22850**
Surinder Sachar
Registration No. 34,423

SIGNATURE

Norman F. Oblon

NAME

24,618

REGISTRATION NUMBER

DATE

Sept. 10 2001

09/936179

213701US-0 PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :
SATORU OKAMOTO ET AL : ATTN: APPLICATION DIVISION
SERIAL NO: NEW US PCT APPLN :
(Based on PCT/JP/00/01478)
FILED: HERewith :
FOR: METHOD FOR SCREENING :
BIOMOLECULE ACTIVITY
REGULATOR

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows:

IN THE SPECIFICATION

Please replace the paragraph beginning on Page 20, line 26 through Page 21, line 16.

A peptide including substitution, insertion and/or deletion of one or more amino acid residues in the amino acid sequence can be easily prepared by using a DNA coding for the peptide specified by the aforementioned amino acid sequence. For example, such a peptide can be obtained by mutating a transformant such as *Escherichia coli* to which a recombinant vector containing the DNA is introduced, by using an agent such as N-methyl-N'-nitro-N-nitrosoguanidine and collecting a gene DNA from microbial cells. The DNA may also be directly treated with an agent such as sodium nitrite. Furthermore, for example, the site-directed mutagenesis (Kramer, W. and Frits, H.J., Methods in Enzymology, 154, 350, 1987),

09936179-001004

the recombinant PCR method described in "PCR Experiment Manual", 155-160, HJB Publications, 1991, the method for preparing mutant genes by PCR described in "Jikken Igaku", Special Issue, 8 (9), 63-67, Yodosha Co., Ltd., 1990 and so forth can be used.

REMARKS

The specification has been amended to correct a clerical error. No new matter is believed to have been added. An action on the merits and allowance of the claims is solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



Norman F. Oblon
Attorney of Record
Registration No. 24,618

Daniel J. Pereira
Registration No. 45,518



22850

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Fax #: (703) 413-2220
DJPER/sej

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Marked-Up Copy
Serial No: New Appl.

Amendment Filed on:

09/10/01

IN THE SPECIFICATION

Please replace the paragraph beginning on Page 20, line 26 through Page 21, line 16.

--A peptide including substitution, insertion and/or deletion of one or more amino acid residues in the amino acid sequence can be easily prepared by using a DNA coding for the peptide specified by the aforementioned amino acid sequence. For example, such a peptide can be obtained by mutating a transformant such as *Escherichia coli* to which a recombinant vector containing the DNA is introduced, by using an agent such as [N-nitro-N'-nitro-N-nitrosoquanidine] N-methyl-N'-nitro-N-nitrosoquanidine and collecting a gene DNA from microbial cells. The DNA may also be directly treated with an agent such as sodium nitrite. Furthermore, for example, the site-directed mutagenesis (Kramer, W. and Frits, H.J., Methods in Enzymology, 154, 350, 1987), the recombinant PCR method described in "PCR Experiment Manual", 155-160, HJB Publications, 1991, the method for preparing mutant genes by PCR described in "Jikken Igaku", Special Issue, 8 (9), 63-67, Yodosha Co., Ltd., 1990 and so forth can be used.--

09/936179

213701US0 PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :
SATORU OKAMOTO, ET AL. : ATTN: APPLICATION DIVISION
SERIAL NO: 09/936,179 :
FILED: SEPTEMBER 10, 2001 :
FOR: METHOD FOR SCREENING
BIOMOLECULE ACTIVITY
REGULATOR

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Responsive to the Official Correspondence dated October 25, 2001, Applicants submit herewith a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. Prior to examination on the merits, please amend the above-identified application as follows.

IN THE SPECIFICATION

Page 54 (Abstract), after the last line, beginning on the next page, please delete the original Sequence Listing attached at pages 1 to 6 and replace it with the substitute Sequence Listing attached hereto.

09/936179-104004

REMARKS

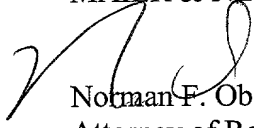
Claims 1-6 are active in the present application.

Applicants have now submitted a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. Contents of the paper copy of the substitute Sequence Listing and the computer-readable Sequence Listing are identical. Support for all the sequences listed in the substitute Sequence Listing can be found in the present application. No new matter is introduced by the submission of the substitute Sequence Listing and the computer-readable Sequence Listing.

Applicants submit that this application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.


Norman F. Oblon
Attorney of Record
Registration No. 24,618

Daniel J. Pereira, Ph.D.
Registration No. 45,518



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09/936179

SEQUENCE LISTING

<110> Okamoto, Satoru
Miwa, Kiyoshi
Eto, Yuzuru

<120> Method For Screening Biomolecule Activity Regulator

<130> 213701US0PCT

<140> US 09/936,179

<141> 2001-09-10

<150> JP99/11-63110

<151> 1999-03-10

<160> 20

<170> PatentIn version 3.1

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<213> ARTIFICIAL SEQUENCE

<220>
<223> SYNTHETIC PEPTIDE

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1

13 Rec'd PCT/PTO 11 MAR 2002

09/936179

DOCKET NO.: 213701US0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF

:

Satoru OKAMOTO, et al.

: ATTN: BOX SEQUENCE

SERIAL NO: 09/936,179

:

FILED: SEPTEMBER 10, 2001

:

FOR: METHOD FOR SCREENING BIOMOLECULE ACTIVITY REGULATOR

PRELIMINARY AMENDMENT AND STATEMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

In response to the Office Communication mailed February 11, 2002, please amend the above-identified application as follows:

IN THE SPECIFICATION

Please amend the specification as follows:

Please replace the paragraph at page 13, lines 3-13, as follows:

When a phage random peptide library is prepared, it is preferred that the peptide presented by phage has a length of 3 to 15 residues. The peptide presented by phage may be directly presented by a surface layer protein of a phage particle or may be presented via a spacer having an appropriate length. Examples of the sequence of the spacer include an amino acid sequence known to successfully present a protein, such as GGGs (SEQ ID NO:32), or a peptide sequence having functions as both of an epitope and a spacer, such as an antibody epitope sequence.

09/936179-0001



Please replace the paragraph at page 29, lines 20-27, as follows:

The peptides are not only presented in a straight-chain form (in an unconstrained or linear form), but also can be expressed in a form having a loop structure (in a constrained or loop form) by providing two cysteine residues at both ends of the sequence to form a disulfide bond. Accordingly, the peptide represented by CX₅C can be expressed by an oligonucleotide having the sequence TGT(NNK)₅TGT (SEQ ID NO:29).

Please replace the paragraph at page 29, line 28 to page 30, line 22, as follows:

PCR amplification was performed by using 5 µg of a synthetic oligonucleotide with a biotinylated 5' end, 5'-ACTCGGCCGACGGGGC-3' (SEQ ID NO: 15), and 5 µg of non-labeled synthetic oligonucleotide, 5'-TTCGGCCCCAGCGGCCCC-3' (SEQ ID NO: 16), as primers and 1 µg of synthetic oligonucleotide, 5'-ACTCGGCCGACGGGGCT(NNK)_nGGGGCCGCTGGGGCCGAA-3' (n = 4 to 15) (SEQ ID NO: 17-28), as a template to obtain a double strand. Taq DNA polymerase (Takara Shuzo) and an attached reaction buffer were used for PCR. A cycle composed of 95°C for 2.5 minutes, 50°C for 4 minutes and 72°C for 2.5 minutes was repeated five times in total to perform amplification and treatment at 72°C for 5 minutes was finally conducted. This PCR product was precipitated with ethanol and the obtained DNA was treated with 200 units each of *Nco*I and *Not*I (Takara Shuzo) at 37°C for 16 hours. Then, the biotinylated DNA fragment was removed by using Streptavidin Agarose (GIBCO BRL). The solution was subjected to phenol/chloroform extraction and chloroform extraction and DNA was precipitated with isopropanol. The obtained DNA was dissolved in sterilised water to obtain random insert DNA.

Please replace the paragraph at page 43, line 23 to page 44, line 15, as follows:

Plasmid was prepared from 950 µl of culture solution by using a FlexiPrep kit (Amersham Pharmacia Biotech). 2.5 µl (about 100 ng) of DNA corresponding to the phage identified as described above among the phagemid DNA obtained in <4> and 1.6 pmol of sequencing primer 5'-TGAATTTTCTGTATGGGG-3' (SEQ ID NO: 30) prepared so that it should bind to a position about 60 nucleotide upstream from the random DNA region on the phage were mixed. A cycle sequencing reaction was performed by using a prism DNA Cycle Sequencing Kit (PE Biosystems) and a PCR apparatus, PCR 9600 (Perkin-Elmer), according to the attached protocol. After the completion of the reaction, the reaction product was collected by ethanol precipitation and dissolved in formamide. Each DNA sequence was determined by using a model 377 DNA sequences (ABI) and the amino acid sequence in the random region of the phage was analyzed. The amino acid sequences and appearance frequency are shown in Table 1.

Please replace the paragraph at page 47, line 7 to page 48, line 16, as follows:

A possibility that an action site of each phage peptide clone obtained as described above and that of the NS4a peptide derived from virus known to be activated by the binding to the NS3 protein were identical or overlapped was examined. A partial peptide of the NS4a peptide composed of the 18th to 40th residues from the amino terminus (referred to as 4A18-40 peptide) is known as a minimal region required to enhance the NS3 protease activity (Tanji et al., J. Virol., 69, 4017-4026, 1995). Accordingly, an influence on the binding of each phage peptide and the MBP-NS34a protein was analyzed by the phage ELISA method using a chemically synthesized 4A18-40 peptide (LTTGSVVIVGRIILSGRPAVVPD, SEQ ID NO: 31). 10 µg/ml of the MBP-NS34a protein to which the 4A18-40 peptide was added in an amount of 10 or 20 times in excess in a molar ratio was immobilized on each well of a 96-well microtiter plate. The plate was blocked by using 100 µl of 0.5% BSA solution prepared

with 10 mM NaHCO₃, and rinsed once with 200 µl of TBS buffer. 100 µl of the phage solution prepared in Example 2, <4> was added to each well of the 96-well plate on which the NS4a peptide and the MBP-NS34a protein were immobilized, and incubated overnight at 4°C. Each well was washed with 200 µl of TBS buffer four times. Then, 50 µl of horseradish peroxidase (HRP)-conjugated sheep anti-M13 phage antibodies diluted 2000 times with 5% skim milk was introduced into each well and incubated at room temperature for 1 hour.

Page 54 (Abstract), after the last line, beginning on the next page, please delete the substitute Sequence Listing filed December 26, 2001, and replace it with the substitute Sequence Listing attached hereto.

REMARKS

Claims 1-6 are active in the present application.

Applicants have now submitted a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. The sequence information recorded in the corresponding computer-readable Sequence Listing is identical to the paper copy of the substitute Sequence Listing. Support for all of the sequences listed in the substitute Sequence Listing is found in the present application as originally filed. No new matter is believed to have been introduced by the submission of the substitute Sequence Listing and the corresponding computer-readable Sequence Listing.

Applicants submit that the present application is ready for examination on the merits.

Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



Norman F. Oblon
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Vincent K. Shier, Ph.D.
Registration No. 50,552



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NFO:VKS:kh

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MARKED-UP COPY

IN THE SPECIFICATION

Please amend the specification as follows:

Please replace the paragraph at page 13, lines 3-13, as follows:

--When a phage random peptide library is prepared, it is preferred that the peptide presented by phage has a length of 3 to 15 residues. The peptide presented by phage may be directly presented by a surface layer protein of a phage particle or may be presented via a spacer having an appropriate length. Examples of the sequence of the spacer include an amino acid sequence known to successfully present a protein, such as GGGG (SEQ ID NO:[20] 32), or a peptide sequence having functions as both of an epitope and a spacer, such as an antibody epitope sequence.--

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primers and 1 µg of synthetic oligonucleotide, 5'-

ACTCGGCCGACGGGGCT(NNK)_nGGGGCCGCTGGGGCCGAA-3' (n = 4 to 15) (SEQ ID NO: 17-28), as a template to obtain a double strand. Taq DNA polymerase (Takara Shuzo) and an attached reaction buffer were used for PCR. A cycle composed of 95°C for 2.5 minutes, 50°C for 4 minutes and 72°C for 2.5 minutes was repeated five times in total to perform amplification and treatment at 72°C for 5 minutes was finally conducted. This PCR product was precipitated with ethanol and the obtained DNA was treated with 200 units each of *Nco*I and *Not*I (Takara Shuzo) at 37°C for 16 hours. Then, the biotinylated DNA fragment was removed by using Streptavidin Agarose (GIBCO BRL). The solution was subjected to phenol/chloroform extraction and chloroform extraction and DNA was precipitated with isopropanol. The obtained DNA was dissolved in sterilised water to obtain random insert DNA.--

Please replace the paragraph at page 43, line 23 to page 44, line 15, as follows:

--Plasmid was prepared from 950 µl of culture solution by using a FlexiPrep kit (Amersham Pharmacia Biotech). 2.5 µl (about 100 ng) of DNA corresponding to the phage identified as described above among the phagemid DNA obtained in <4> and 1.6 pmol of sequencing primer 5'-TGAATTTTCTGTATGGGG-3' (SEQ ID NO: [18]30) prepared so that it should bind to a position about 60 nucleotide upstream from the random DNA region on the phage were mixed. A cycle sequencing reaction was performed by using a prism DNA Cycle Sequencing Kit (PE Biosystems) and a PCR apparatus, PCR 9600 (Perkin-Elmer), according to the attached protocol. After the completion of the reaction, the reaction product was collected by ethanol precipitation and dissolved in formamide. Each DNA sequence was determined by using a model 377 DNA sequences (ABI) and the amino acid sequence in the

random region of the phage was analyzed. The amino acid sequences and appearance frequency are shown in Table 1.--

Please replace the paragraph at page 47, line 7 to page 48, line 16, as follows:

--A possibility that an action site of each phage peptide clone obtained as described above and that of the NS4a peptide derived from virus known to be activated by the binding to the NS3 protein were identical or overlapped was examined. A partial peptide of the NS4a peptide composed of the 18th to 40th residues from the amino terminus (referred to as 4A18-40 peptide) is known as a minimal region required to enhance the NS3 protease activity (Tanji et al., J. Virol., 69, 4017-4026, 1995). Accordingly, an influence on the binding of each phage peptide and the MBP-NS34a protein was analyzed by the phage ELISA method using a chemically synthesized 4A18-40 peptide (LTTGSVVIVGRIILSGRPAVVPD, SEQ ID NO: [19]31). 10 µg/ml of the MBP-NS34a protein to which the 4A18-40 peptide was added in an amount of 10 or 20 times in excess in a molar ratio was immobilized on each well of a 96-well microtiter plate. The plate was blocked by using 100 µl of 0.5% BSA solution prepared with 10 mM NaHCO₃, and rinsed once with 200 µl of TBS buffer. 100 µl of the phage solution prepared in Example 2, <4> was added to each well of the 96-well plate on which the NS4a peptide and the MBP-NS34a protein were immobilized, and incubated overnight at 4°C. Each well was washed with 200 µl of TBS buffer four times. Then, 50 µl of horseradish peroxidase (HRP)-conjugated sheep anti-M13 phage antibodies diluted 2000 times with 5% skim milk was introduced into each well and incubated at room temperature for 1 hour.--

Page 54 (Abstract), after the last line, beginning on the next page, please delete the substitute sequence listing filed December 26, 2001, and replace it with the substitute sequence listing attached hereto.

SEQUENCE LISTING

<110> OKAMOTO, SATORU
MIWA, KIYOSHI
ETO, YUZURU



<120> METHOD FOR SCREENING BIOMOLECULE ACTIVITY REGULATOR

430> 213701US0PCT

440> 09/936,179

441> 2001-09-10

450> JP 99/11-63110

451> 1999-03-10

<160> 32

<170> PatentIn version 3.1

<210> 1

<211> 10

<212> PRT

<213> ARTIFICIAL SEQUENCE

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Cys Val Pro Leu Val Cys Ile Phe Arg Cys
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<210> 2

<211> 10

<212> PRT

<213> ARTIFICIAL SEQUENCE

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<223> Synthetic Peptide

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<210> 3

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<212> PRT

<213> ARTIFICIAL SEQUENCE

<220>

<223> Synthetic Peptide

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Cys Trp Leu Phe Leu Trp Cys
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<210> 4

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<213> ARTIFICIAL SEQUENCE

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<210> 7

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<210> 8

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400T60 6CTEE60

4/PRTS

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1

METHOD FOR SCREENING BIOMOLECULE ACTIVITY REGULATOR

Technical Field

The present invention relates to a method for screening a substance regulating an activity of a biomolecule. In particular, the present invention provides a method useful for screening of a compound that may be used as, for example, a starting material of a drug.

Background Art

Lead compounds, which are used as starting materials for development of drugs, have been screened by assaying, in a test tube, secondary metabolites produced by microorganisms or low molecular weight compounds chemically synthesized by referring to known molecular structures, or evaluating such substances by utilizing cells into which a reporter gene or the like is incorporated. Recently, a large scale random screening has been started by drug manufacturers by combining the high throughput screening, in which mechatronics technologies are applied to drug screening so that a system for the aforementioned assay is automatically operated in a short time, and the combinatorial chemistry technique, in which various compounds are automatically synthesized.

In recent years, a method has been known in

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which a collection of organisms expressing and presenting random amino acid sequences, that is, a peptide library, is constructed and sequences that can bind to a specific biomolecule are selected from this library. As such a library, the phage display random peptide library using a coat protein of filamentous phage has been reported (Scott, J.K. et al., Science, 249: 386-390, 1990). Libraries using such organisms and chemically synthesized libraries prepared on solid phase supports (Lam, K.S. et al., Nature, 354, 82, 1991) have been established as means for retrieving lead peptide compounds of drugs. For example, the aforementioned phage display random peptide library is used to retrieve lead peptide compounds useful in development of drugs such as hormone-mimic peptides (Wrighton, N.C. et al., Science, 273, 458, 1996 and Cwirla, S.E. et al., Science, 276, 1696, 1997) and receptor-ligand binding inhibitors (Martens, C.L. et al., J. Biol. Chem., 270, 21129, 1995).

However, while bioactivity of peptides retrieved from these peptide libraries can be improved by addition, substitution or the like of an amino acid so that an efficacy is exhibited even with a lower dosage, various problems mentioned below are expected when the peptides are used as therapeutic agents as they are. For example, problems often arise that bioavailability is low due to their fast metabolism in a living body, that administration conditions as a drug are limited

due to their poor solubility, that peptides are easily recognized by the immune system due to their relatively high molecular weight and so forth. That is, it is readily expected that pharmacological actions of peptides as they are in humans would be low and thus it would be extremely difficult to develop drugs with such peptides. To overcome these problems, replacement of peptides with low molecular weight non-peptidic compounds that mimic three-dimensional structures of the peptides has long been attempted. However, this method is technically very difficult and there has been no report on examples in which practical drugs are successfully developed.

Meanwhile, as a method for identifying ligands that can bind to at least one determinant in a biologically active site on a target, a method using a reporter antibody selected from a divergent antibody library is known (Japanese Patent Unexamined Publication in Japanese (KOHYO) No. 10-507517). In this method, a target substance (ligand) is screened by using a variable region of an antibody selected from a phage library by detecting binding to a specific protein as an index. The binding affinity of an antigen protein and an antibody protein is considerably strong due to their large binding region. Therefore, when a library of compounds having a low molecular weight of several hundred daltons on average is screened for active substances by using the

antibody-presenting phage as a probe and the degree of inhibition for binding to the target protein as an index, it is anticipated that competitive substances cannot be retrieved unless the substances can very strongly interact by themselves. Further, it is also readily expected that it is highly possible that substances that bind to regions unrelated to the protein function are selected since the antibody molecules recognize complicated structures of proteins as their characteristics.

Disclosure of the Invention

The present invention was accomplished in view of the current situation described above, and an object thereof is to provide a method for screening a low molecular weight compound free from the problems described above, which is a substance that binds to a specific site of a biomolecule and thus may be used as a starting material in development of drugs.

To achieve the aforementioned object, the inventors of the present invention selected phage presenting relatively short random peptides for screening of a lead compound. That is, they considered that peptides having a molecular weight close to that of usual drugs (about several hundreds) would be more suitable to search for a further limited region (hot spot) within the functional region involved in an interaction between biomolecules. In other words, they

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considered that it would be advantageous to screen various biomolecule activity regulators having low molecular weights by using a peptide presented by phage as a probe because of its binding affinity tens to hundreds times lower (micromole (μM) order) than that of the antibody presented by phage. Further, it was also expected that a binding of low molecular weight peptide and a biomolecule that was irrelevant to the biomolecule function would be more unlikely to be selected compared with a case where an antibody molecule was used.

It has been shown that methods for selecting peptide compounds by utilizing an interaction between molecules as an index, of which typical example is the method utilizing a phage display random peptide library, are useful to retrieve peptide molecules inhibiting interactions between proteins or peptide molecules mimicking cytokine activities. Further, in general, interactions between proteins such as cleavage of a substrate protein by a protease, phosphorylation of a specific substrate molecule by a phosphorylating enzyme, and binding of a receptor present on a cell surface and a ligand are attained by characteristic amino acid residues exposed and localized on the surface of the proteins, specifically, amino acids having positively or negatively charged side chains or hydrophobic side chains. The present inventors found from the results of their various

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researches using phage display random peptide libraries that it was highly possible that peptides having an ability to bind to a specific protein selected from a phage display random peptide library would interact with an important functional region of the protein and that identification of such a peptide was equivalent to elucidation of the functional region of the protein.

Meanwhile, it is evident that serine protease, nonstructural protein 3 (hereafter, abbreviated as "NS3 protease") or cpro-2 encoded in the genome of human hepatitis C virus (hereafter, abbreviated as "HCV") plays an important role in proliferation of the virus (refer to Patick, A.K. et al., Clinical Microbiology Reviews, 11 (4), 614-627, 1998 and so forth). Since a drug inhibiting this NS3 protease activity is considered to be effective in prevention or treatment of various liver diseases induced by infection and proliferation of HCV, for example, cirrhosis and liver cancer, such a drug is being searched for around the world, but no promising drug has been found yet. The present inventors obtained the aforementioned conception while searching for a drug inhibiting the NS3 protease. Then, they found a plurality of phage clones having affinity for the NS3 protease from phage random peptide libraries. They also found that these oligopeptides inhibited a substrate cleavage reaction by the NS3 protease.

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Moreover, they found a drug inhibiting the binding of the NS3 protease to phage presenting the oligopeptides, from chemical libraries by an ELISA system for detecting the binding and showed that this drug could inhibit the NS3 protease activity. The present invention was accomplished based on these findings.

That is, the present invention provides a method for screening a substance which interacts with a specific region of a biomolecule having an activity, to regulate the activity, said method comprising the following steps:

- (a) a step of selecting a recombinant organism that interacts with the biomolecule from a peptide library composed of a collection of recombinant organisms each presenting at least one of various peptides on its surface with a proviso that the interaction is not an antigen-antibody reaction; and
- (b) a step of selecting a substance inhibiting the interaction between the selected recombinant organism or a peptide presented by the recombinant organism and the biomolecule.

In a preferred embodiment of the present invention, the biomolecule is a protein, a nucleic acid or a sugar chain.

In a preferred embodiment of the present invention, the peptide library is composed of random peptide-presenting phage or random peptide-presenting *Escherichia coli*.

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In a preferred embodiment of the present invention, the peptides each have a length of 3 to 15 residues.

In a preferred embodiment of the present invention, the selected recombinant organism or the peptide presented by the recombinant organism is labeled with a labeling substance.

A substance selected by the method of the present invention can be used as a low molecular weight lead compound of a drug because the substance can interact with a specific region of a biomolecule such as a protein, a nucleic acid or a sugar chain to regulate its activity.

In the present invention, the "interaction" means that a molecule in a living body approaches another biomolecule to exhibit its function and induces a certain change in each of them. The expression of "regulating an activity of a biomolecule" includes reducing, eliminating or increasing the activity of the biomolecule, or reducing, eliminating or increasing activation or inactivation of the biomolecule.

The present inventors found from results of screening of peptides binding to a target protein using phage peptide libraries that most of the selected peptides bound to a region involved in the target protein function and few peptides were selected based on meaningless binding. For example, when a

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phage peptide library was screened by using IgG protein in order to select an epitope peptide of an antibody, peptide sequences that specifically bind to regions involved in other functions such as Fc regions of the IgG protein, i.e., an Fc receptor-binding site, and a complement protein-binding site, were selected in addition to the peptide sequence recognized and selected as the epitope.

Further, the maltose-binding protein (MBP) is widely utilized as a tag protein because it can be stably expressed in a large amount in *Escherichia coli* and purified by simple affinity purification. When binding peptides are selected by using a protein fused with this protein, peptide sequences specifically binding to MBP were also selected in addition to peptides specifically binding to the fused protein. That is, selection of peptide binding to a specific protein from a phage peptide library is exactly the same as searching of a peptide binding to the functional domain of the protein.

The following possibilities are implied from our results. If the protein is a receptor, a peptide binding to an extracellular ligand-binding site or an intracellular signal-transmitting site may be selected. If the protein is a ligand, a peptide binding to a receptor-binding site or a dimer-forming site may be selected. If the protein is an enzyme, a peptide binding to a substrate-recognizing site or an

activator-binding site may be selected. If the protein is a transcription factor, a peptide binding to a DNA-binding site, a ligand-recognizing site or sites involved in interactions with other transcription factors may be collected.

Specific examples of the biomolecule to which the method of the present invention is applicable include NS3 protease of HCV, integrin, matrix metalloprotease, selectin and so forth.

As described above, a peptide that interacts with a biomolecule selected from a peptide library binds to a region involved in the function of the biomolecule. Therefore, it is highly possible that a substance inhibiting such an interaction between the peptide and the biomolecule also interacts with the biomolecule and binds to a region involved in the function. This strongly suggests that the function (activity) of the biomolecule can be regulated.

The present inventors designated a peptide binding to a specific region (functional region) of a protein as a surrogate peptide. That is, in the method provided by the present invention, as a first step, a phage presenting a peptide molecule that binds to a functional region of a protein, that is, a surrogate peptide, is retrieved by using a library composed of various molecules, for example, a phage random peptide library, and as a second step, a compound inhibiting the interaction between the protein and the surrogate

phage is selected from various compound libraries. Then, as a third step, which is optionally performed, a biochemical activity of the selected compound, that is, enzyme inhibition activity or activity for inhibiting binding of the protein is biochemically evaluated by using an appropriate assay. By using the above-described method, a method for efficiently selecting compounds having an activity for regulating an interaction between proteins can be provided.

Brief Description of the Drawings

Fig. 1 shows affinities for MBP-NS34a of 12 kinds of phage clones having peptides selected according to the present invention, which were determined by the ELISA method.

Fig. 2 shows effect of addition of the NS4a peptide on the binding of a phage having peptide selected according to the present invention to MBP-NS34a, which were determined by the phage ELISA method. Binding of the phage peptide K13 was inhibited the binding in a concentration-dependent manner.

Fig. 3 shows effect of the protease inhibitor HCP1271 on the binding of various phages to NS34a, which were determined by the phage ELISA method. Binding of clones other than the phage peptide K13 was inhibited in a concentration-dependent manner.

Fig. 4 shows effect of the protease inhibitor HCP1231 on the binding of various phages to NS34a,

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can form a phage particle, but a phagemid vector is preferred.

When a phage random peptide library is prepared, it is preferred that the peptide presented by phage has a length of 3 to 15 residues. The peptide presented by phage may be directly presented by a surface layer protein of a phage particle or may be presented via a spacer having an appropriate length. Examples of the sequence of the spacer include an amino acid sequence known to successfully present a protein, such as GGGS (SEQ ID NO: 20), or a peptide sequence having functions as both of an epitope and a spacer, such as an antibody epitope sequence.

As the random peptide-presenting *Escherichia coli* library, a library in which random peptides are presented on the surface layer of flagellin proteins, which constitute a flagellum of *Escherichia coli*, is commercially available from Invitrogen and usable. The peptide presented by *Escherichia coli* preferably has a length of 3 to 15 residues.

Hereafter, a case where a target biomolecule is the NS3 protease will be mainly explained, but the biomolecule in the present invention is not limited to the NS3 protease. When the present invention is applied to another biomolecule, the NS3 protease and related items can be replaced with the target biomolecule and items therefor in the following description. The following explanation concerns a case

where random peptide-presenting phage is used as a peptide library, but the peptide library used in the present invention is not limited to the random peptide-presenting phage.

First, to select a phage that binds to the target NS3 protease, the NS3 protease is adsorbed to, for example, a tube or a plate and the aforementioned library is brought into contact with the NS3 protease. Then, non-binding phages are washed away to leave the binding phages. After the washing, the remaining phages are eluted with an acid solution of about pH 2 or the like. Then, the eluate is neutralized and *Escherichia coli* is infected with the phages to amplify the phages. By repeating this selection process for a plurality of times, a plurality of phages having affinity for the target NS3 protease are concentrated. Then, to obtain a single clone, *Escherichia coli* is reinfected with the phages and the bacterium is allowed to form colonies on an agar medium. Each colony is cultured in a liquid medium and then phages present in the medium supernatant are precipitated and purified with polyethylene glycol or the like. DNA is prepared from the obtained phages and the structure of the peptide can be obtained by analyzing the nucleotide sequence of the DNA.

As the method for preparing a peptide library having random amino acid sequences, chemically synthesized peptides can also be used in addition to

the above-described method using phages. As examples of such a method, there have been reported the method using beads (Lam, K.S. et al., Nature, 354, 82, 1991), the liquid phase focusing method (Houghton, R.A. et al., Nature, 354, 84, 1991), the microplate method (Fodor, S.P.A. et al., Science, 251, 767, 1991) and so forth. As protection groups such as one for an amino group in the peptide synthesis and condensing agents for condensation reaction, for example, those described in "Protein Engineering: Fundamentals and Applications", Ed. by Suzuki, K., Maruzen Co., Ltd., 1992; "Peptide Synthesis", Bodanszky M., et al, John Wiley & Sons, N.Y. 1976 and "Solid Phase Peptide Synthesis", Stewart, J.M. et al., W.H. Freeman and Co., San Francisco, 1969 and so forth can be used. For the solid phase method, various commercially available peptide synthesizers can be used.

The method for preparing peptides used in the present invention is not limited to the aforementioned chemical or biological methods.

To determine whether the obtained peptides inhibit the NS3 protease activity, for example, a peptide that can be digested by the NS3 protease as a substrate is prepared by a recombination method or a chemical synthesis method, labeled with fluorescence, radioisotope or the like and mixed with the NS3 protease in the presence of the peptide to be evaluated. Degree of inhibition for the digestion of

the substrate compared with the case where the peptide to be evaluated is not added can be determined by examining the digestion of the substrate through liquid chromatography or electrophoresis.

Specifically, a gene coding for the amino acid sequence (985th to 1647th amino acid residues) in a polyprotein encoded by the HCV genome can be expressed and digestion of the substrate performed by using the obtained NS3 protease can be detected by liquid chromatography according to the method of Kakiuchi et al. (Biochem. Biophys. Res. Commun., 210, 1059, 1995).

Phages having affinity for the NS3 protease can also be screened by the method of Smith et al. (Smith G.P. et al., *Methods in Enzymology*, 217, 228-257). That is, a protein containing the catalytic domain of the NS3 protease obtained by expressing the gene coding for the NS3 protease as described above can be immobilized on a well of a microtiter plate and phages binding to the NS3 protease can be detected.

More precisely, DNA coding for the catalytic domain of the NS3 protease (catalytic region protein) is obtained based on the information of the HCV genome sequence. Desired DNA can be easily obtained by the PCR (polymerase chain reaction) method. At this time, a specific protein is preferably fused to the N-terminus or C-terminus of the protein to simplify the purification. As such a protein, the maltose-binding protein (MBP) or the like can be mentioned.

For example, since the NS3 protease is expressed in a patient infected with human hepatitis C virus, a sense chain oligonucleotide primer and an anti-sense chain oligonucleotide primer can be prepared based on the amino acid sequence coding for the region having the NS3 protease activity, and a DNA coding for the catalytic region protein of the NS3 protease can be amplified by PCR using these primers and cDNA prepared from a sample derived from the patient as a template. Alternatively, the DNA can also be obtained by total chemical synthesis. Further, when MBP is added, for example, there can be used a primer obtained by ligating an anti-sense DNA of the DNA coding for MBP to the sense primer of the catalytic region protein of the NS3 protease so that their reading frames match each other.

Thus, a DNA fragment coding for the catalytic domain of the NS3 protease, for example, a DNA fragment coding for the NS3 protease catalytic region protein fused with MBP (hereafter, also abbreviated as "MBP-NS34a" in the present specification) can be obtained and a recombinant vector for expressing the DNA can be prepared by incorporating the DNA fragment into a vector. The kind of the vector is not particularly limited so long as it can express the DNA in animal cells, plant cells, microorganism cells or the like. As for the MBP-fused protein, a substantial amount of the protein can be obtained by using a

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can be amplified.

The phage can be obtained from a culture supernatant obtained by infecting the infected *Escherichia coli* with a M13K07 helper phage and culturing it overnight, by precipitation with polyethylene glycol. By repeating similar screening with the amplified phage, for example, a phage that firmly binds to MBP-NS34a can be concentrated.

A phage that may bind to MBP-NS34a, for example, can easily be prepared by culturing an *Escherichia coli* colony on an ampicillin plate obtained by the above method and infecting the bacteria with a helper phage such as M13K07. For example, a phage can be prepared by culturing an *Escherichia coli* colony on the ampicillin plate, infecting the bacteria with a helper phage such as M13K07, culturing them overnight, centrifuging the culture solution and subjecting the supernatant to precipitation with polyethylene glycol. Whether the obtained phage clone binds to, for example, MBP-NS34a or not can be determined by the ELISA method utilizing an antibody recognizing that phage. For example, a phage clone coding for a peptide having affinity for MBP-NS34a can be finally obtained by using the HRP/anti-M13 conjugate (Amersham Pharmacia Biotech).

From the phage clone that has been thus confirmed to bind to MBP-NS34a, a double-stranded DNA derived from the selected phage can be obtained by

using a FlexiPrep Plasmid extraction Kit (Amersham Pharmacia Biotech) or the like. For example, a random DNA region can be determined from the phage DNA that has been confirmed to bind to MBP-NS34a by the method of Sanger et al. (Sanger, F. et al., Proc. Natl. Acad. Sci. USA, 74, 5463, 1977), for example. The amino acid sequence specifically presented by the phage clone can be easily deduced from the sequence of this DNA region.

Standard operations required for handling of phage, DNA, and *Escherichia coli* as a recombination host described in the present specification are well known to those skilled in the art and described in, for example, the laboratory manual of Maniatis et al. (Maniatis T. et al., Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, 1989). As all the enzymes and reagents to be used, commercially available products can be used. Usually, the products can be used according to their specified conditions to completely fulfill their purposes. In addition to the above general explanations, methods for biologically preparing peptides used in the present invention are specifically explained in detail in the examples of the present specification. However, the method for biologically preparing peptides used in the present invention is not limited to these methods.

A peptide including substitution, insertion and/or deletion of one or more amino acid residues in the amino acid sequence can be easily prepared by

using a DNA coding for the peptide specified by the aforementioned amino acid sequence. For example, such a peptide can be obtained by mutating a transformant such as *Escherichia coli* to which a recombinant vector containing the DNA is introduced, by using an agent such as N-nitro-N'-nitro-N-nitrosoguanidine and collecting a gene DNA from microbial cells. The DNA may also be directly treated with an agent such as sodium nitrite. Furthermore, for example, the site-directed mutagenesis (Kramer, W. and Frits, H.J., Methods in Enzymology, 154, 350, 1987), the recombinant PCR method described in "PCR Experiment Manual", 155-160, HJB Publications, 1991, the method for preparing mutant genes by PCR described in "Jikken Igaku", Special Issue, 8 (9), 63-67, Yodosha Co., Ltd., 1990 and so forth can be used.

A substance inhibiting an interaction between the NS3 protease and a peptide, that is, a substance that interacts with the NS3 protease, can be screened by using the phage peptide obtained as described above. That is, a compound inhibiting binding of the phage peptide and the NS3 protease can be identified as follows. The NS3 protease is immobilized on, for example, a 96-well multi-titer plate or the like and various compounds and phage peptides appropriately prepared are each added to each well of the plate, and incubated. After washing, the amount of the phage left in each well is detected by using, for example, a

horseradish peroxidase-conjugated goat anti-M13 antibody to identify a compound inhibiting binding of each phage peptide and the NS3 protease. Alternatively, if each phage peptide is labeled with a lanthanide compound such as europium in advance, the operation becomes simpler, and it is suitable for high throughput screening of many kinds of compounds according to the present invention. The phage peptide may also be directly labeled with a labeling substance.

As described above, it is preferable to label a recombinant organism or a peptide presented thereby with a labeling substance in the present invention. The labeling scheme may be direct labeling or indirect labeling through binding of a substance that is directly labeled with a labeling substance and specifically recognizes the recombinant organism or the like. A target biomolecule may also be labeled.

Examples of the labeling substance include radioisotopes, fluorescent substances, enzymes, biotin, avidin, streptavidin and lanthanide compounds such as europium and so forth. Examples of the radioisotopes include ^3H , ^{14}C , ^{32}P , ^{35}S , ^{125}I and so forth. Examples of the fluorescent substances include FITC, TRITC, DTAF and so forth. Examples of the enzymes include peroxidase, β -galactosidase, alkaline phosphatase and so forth. For labeling and detection using these labeling substances, the conventional ELISA method known to those skilled in the art can be used.

When it is confirmed that a substance inhibiting an interaction between a target biomolecule and a surrogate peptide selected as described above can regulate the activity of the biomolecule, the substance can be a lead compound used as a starting material in development of drugs.

Substances selected by the present invention can be made into substances having a higher biological activity by modifying them with various methods.

Specific examples of the peptide that binds to the NS3 protease include the peptides specified by the amino acid sequences of SEQ ID NOS: 1 to 12 obtained in the examples and the corresponding peptides having a cyclic structure, in which two cysteine residues contained in each amino acid sequence forms a disulfide-bond. In the above sequences, the amino acid residue may be either an L- or D-amino acid residue, preferably an L-amino acid residue. The peptides according to the present invention have two cysteine residues, which may form a disulfide-bond to form a cyclic structure. Such cyclic peptides are of course encompassed in the scope of the peptides used in the present invention. Although the present invention is not bound by any specific theory, but it is possible that the above-described cyclic structure is essential for the peptides used in the present invention to express a desired physiological activity. Therefore, in such a case, the peptide having the cyclic

A peptide including substitution, insertion, and/or deletion of one or more amino acid residues in any of the aforementioned amino acid sequences and having affinity for the NS3 protease or a corresponding peptide having a cyclic structure, in which two of the cysteine residues contained in the peptide form a disulfide bond, are also provided by the present invention. The kind of one or more substituted and/or inserted amino acids is not particularly limited, but an L-amino acid is preferred. Whether a peptide including substitution, insertion and/or deletion of one or more amino acid residues substantially has affinity for the NS3 protease or not can easily be confirmed by those skilled in the art according to the methods described in the examples.

According to yet another aspect of the present invention, there are provided a drug containing as an active ingredient any of the above peptides or substances which are selected by the method of the present invention using these peptides and which interact with the NS3 protease, preferably a drug in the form of a pharmaceutical composition containing any of the above peptides or the interacting substances and additives for pharmaceutical preparations. Since these peptides or the interacting substances can inhibit the NS3 protease activity, the

aforementioned drug is useful for treatment, prevention or diagnosis of diseases of mammals including humans induced by abnormality of liver cells due to infection and proliferation of hepatitis C virus, particularly, diseases accompanied with degeneration of liver cells. Examples of such diseases include cirrhosis, liver cancer and so forth, but the diseases are not limited to these ones.

One or more kinds of peptides selected from the above peptides may be used as they are as the above drug. However, it is usually preferred that a pharmaceutical composition containing one or more kinds of the above peptides as an active ingredient is prepared by using one or more kinds of pharmaceutically acceptable additives for pharmaceutical preparations, and used for treatment and/or prevention of the aforementioned diseases. In view of pharmacokinetics such as solubility, absorption and excretion and/or formulation methods, the peptides may be in the form of a physiologically acceptable salt. Examples of administration routes of the pharmaceutical composition include systemic administration such as intravenous administration, intrarectal administration and oral administration, as well as local administration such as external use, instillation of eye drop, nasal drop or ear drop, and local injection.

For example, an agent for systemic

administration such as injection for intravenous administration or drip infusion is a preferred form of the pharmaceutical composition. Use of pharmaceutical compositions containing the active ingredient encapsulated in liposomes or the like or bound to antibodies or the like may improve affinity or selectivity for a specific target organ. However, as a matter of course, the administration route can be appropriately selected depending on the kind of the applicable disease, purpose of administration including treatment or prevention, conditions of patients and so forth, and a dosage form suitable for each administration route can also be appropriately selected.

Examples

The present invention will be explained in further detail with reference to the following examples, but the scope of the present invention is not limited to these examples.

Example 1: Preparation of phage library and selection of specifically binding phage peptide by panning method

This example shows a method for preparing a phage library and a method for identifying a phage expressing a peptide binding to a protein in a directed manner, which is selected by screening a

library by using the panning method.

<1> Construction of random peptide-presenting vector pSBSX

A phagemid vector for presenting a single-stranded antibody, pCANTAB5E (available from Amersham Pharmacia), was modified into a vector for presenting random peptides by using synthetic oligonucleotides. As the reagents and enzymes, commercially available products were used. As the synthetic DNA primers, products manufactured by Japan Bio Service were used. Two kinds of oligonucleotides shown below were annealed and inserted into the *Nco*I and *Not*I sites of the pCANTAB-5E vector to prepare the pSBSX vector.

Sense strand:

5'-CATGGCAGATCTTTAAGTCGACTCTAGAGGCCTCTGC-3' (SEQ ID NO: 13)

Anti-sense strand:

5'-GGCCGCAGAGGCCTCTAGAGTCTACTTAAAGATCTGC-3' (SEQ ID NO: 14)

<2> Preparation of vector DNA

A phage random peptide library was prepared by using the pSBSX vector prepared as described above as a raw material, according to the method of Nishi et al. (Jikken Igaku, 11, 13, 1759-1764), the method of Smith et al. (Smith G.P. et al., Science, 249, 386-390, 1990) and the method described by Koivunen et al. (see

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al. (supra) for preparing double-stranded DNA by PCR using 5' end biotinylated primers. Accordingly, 10 kinds of libraries coding for peptides designated as X_6 , X_9 , X_{15} , CX_4C , CX_5C , CX_6C , CX_7C , CX_8C , CX_9C and CX_{12} were prepared. Here, "C" represents cysteine, and " X_n " indicates that a given number (n) of independently selected amino acids are serially linked. These libraries can each present a cyclic peptide when the peptide contains at least two cysteine residues. The oligonucleotides were constructed such that "C" should be encoded by the codon TGT and that " X_n " should be encoded by $(NNK)_n$. Here, "N" is an equimolar mixture of A, C, G and T, and "K" is an equimolar mixture of G and T. The collection of DNAs represented by the NNK includes 32 kinds of combinations, which include codons of all the 20 kinds of amino acids. Degenerate oligonucleotides are synthesized by repeating this NNK the same number of times as the desired number of amino acids.

The peptides are not only presented in a straight-chain form (in an unconstrained or linear form), but also can be expressed in a form having a loop structure (in a constrained or loop form) by providing two cysteine residues at both ends of the sequence to form a disulfide bond. Accordingly, the peptide represented by CX_5C can be expressed by an oligonucleotide having the sequence $TGT(NNK)_5TGT$.

PCR amplification was performed by using 5 μ g of

a synthetic oligonucleotide with a biotinylated 5' end, 5'-ACTCGGCCGACGGGGC-3' (SEQ ID NO: 15), and 5 µg of non-labeled synthetic oligonucleotide, 5'-TTCGGCCCCAGCGGCCCC-3' (SEQ ID NO: 16), as primers and 1 µg of synthetic oligonucleotide, 5'-ACTCGGCCGACGGGGCT(NNK)_nGGGGCCGCTGGGGCCGAA-3' (n = 4 to 15) (SEQ ID NO: 17), as a template to obtain a double strand. Taq DNA polymerase (Takara Shuzo) and an attached reaction buffer were used for PCR. A cycle composed of 95°C for 2.5 minutes, 50°C for 4 minutes and 72°C for 2.5 minutes was repeated five times in total to perform amplification and treatment at 72°C for 5 minutes was finally conducted. This PCR product was precipitated with ethanol and the obtained DNA was treated with 200 units each of NcoI and NotI (Takara Shuzo) at 37°C for 16 hours. Then, the biotinylated DNA fragment was removed by using Streptavidin Agarose (GIBCO BRL). The solution was subjected to phenol/chloroform extraction and chloroform extraction and DNA was precipitated with isopropanol. The obtained DNA was dissolved in sterilized water to obtain random insert DNA.

<4> Ligation of phagemid vector DNA and random insert DNA

The random insert DNA was ligated to the DNA coding for the gene III protein in the pSBSX vector, which was designed such that the peptide encoded by

the random insert DNA should be present at the N-terminus of the gene III protein as a fusion protein upon expression of gene III. 7.45 µg of the pSBSX DNA digested with *Nco*I, *Bgl*III, *Sal*I and *Not*I obtained in <2> and 0.2 µg of the random insert DNA obtained in <3> were subjected to ligation reaction at 16°C for 16 hours by using an isovolume Ligation High Mix (Toyobo). The reaction mixture was subjected to phenol/chloroform extraction and chloroform extraction and DNA was precipitated with ethanol. The obtained DNA was dissolved in sterilized water and then subjected to ultrafiltration by using a Millipore Filter Ultrafree C3 (trade name; Millipore) to be concentrated to a DNA concentration of 5 µg/µl or higher. The obtained solution was stored at -20°C until it was used for preparation of a phage random peptide library.

<5> Preparation of phage display random peptide library

Phages were prepared by using the *Escherichia coli* TG1 strain, according to the protocol attached to the product manufactured by Amersham Pharmacia Biotech. *Escherichia coli* was transformed by electroporation. As *Escherichia coli* competent cells, those of the commercially available TG1 strain for electroporation (Amersham Pharmacia Biotech) were used. A required amount of competent cells were thawed on ice and mixed

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together to 200 ml of 2 x YT-AG medium maintained at 37°C. The M13K07 helper phage at an m.o.i. of 10 was added thereto and the mixture was further shaken at 37°C and 150 rpm for 30 minutes. The microbial cells were collected by centrifugation (4°C, 3000 rpm, 15 minutes), resuspended in 400 ml of 2 x YT-AK medium (2 x YT medium containing 100 µg/ml of ampicillin and 50 µg/ml of kanamycin) and cultured at 37°C overnight with shaking at 150 rpm. The culture solution was subjected to centrifugation (4°C, 3000 rpm, 30 minutes) and phage was obtained at a high concentration from this phage culture solution by the method utilizing precipitation with polyethylene glycol (PEG). A PEG/NaCl solution (20% PEG8000 (W/V)/2.5 M NaCl) was added thereto in a 0.15-fold amount and the mixture was left standing overnight at 4°C. Then, the supernatant was removed by centrifugation (4°C, 16000 rpm, 30 minutes) and the precipitate was resuspended in TBS (50 mM Tris-HCl, 150 mM NaCl) buffer in a 0.1-fold amount. The supernatant was collected by centrifugation (4°C, 16 rpm, 15 minutes) and phage particles were precipitated again with the PEG/NaCl solution in a 0.15-fold amount and suspended in the TBS buffer to finally concentrate the phage solution about 100-fold.

<7> Measurement of phage titer

The titer of the obtained phage was calculated

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having the NS3 protease activity was introduced into the vector pMAL-c2 expressing MBP fusion protein (New England Biolabs) so that their reading frames should match, to construct a recombinant vector for expressing the NS3 protease protein. Use of the pMAL bacterial expression vector for the purpose of expressing the MBP fusion protein is well known to those skilled in the art. Subsequently, the obtained DNA was introduced into the *Escherichia coli* XL2-Blue strain (Stratagene) by the calcium chloride method. A strain in which the insertion occurred so that the fusion protein could be expressed, was selected from the obtained transformants.

<9> Expression in large amount and purification of MBP-NS34a

The MBP fusion protein was expressed and purified according to the protocol attached to the aforementioned expression vector. Colonies of the *Escherichia coli* strain prepared as described above formed on LB agar medium containing 100 µg/ml of ampicillin were directly inoculated into five of 500-ml Sakaguchi flasks, each containing 200 ml of LB medium containing 50 µg/ml of ampicillin, that is, 1 L in total, and shaken at 30°C for 14 to 16 hours to be cultured until OD600 reached 0.5 to 0.7. The medium was cooled at 4°C, and IPTG (isopropyl-β-D-thiogalactopyranoside) was added at a final

concentration of 0.5 mM. The culture was further cultured at 20°C for 4 hours with shaking. Then, the microbial cells were collected by centrifugation. The microbial cells were washed with 0.85% NaCl solution and suspended in 50 ml of Buffer A (10 mM Na-phosphate buffer (pH 7.2), 30 mM NaCl, 2 mM DTT (dithiothreitol)). After the solution was frozen and thawed, a supernatant was collected by centrifugation. The collected supernatant was subjected to precipitation with 70% ammonium sulfate and a pellet was collected by centrifugation. The pellet was suspended in Buffer B (10 mM Na-phosphate buffer (pH 7.2), 30 mM NaCl, 10 mM 2-ME (mercaptoethanol), 0.25% Tween 20) and left overnight at 4°C to obtain a crude extract. Subsequently, the extract was applied to an amylose resin column (15 mm in diameter and 10 cm in length) equilibrated with Buffer B, at a flow rate of 0.5 ml/min. This process was repeated several times.

The column was washed with Buffer B of 4 times the column volume and Buffer C (10 mM Na-phosphate buffer, pH 7.8, 0.5 M NaCl) of 8 times the column volume and eluted with Buffer C containing 10 mM maltose to recover a purified enzyme solution. After quantification, glycerol at a concentration of 20% was added to the solution and the mixture was stored at -80°C until use. About 10 mg of purified MBP-NS34a could be obtained from 1 L of the culture.

<10> Quantification of MBP-NS34a by ELISA

1.2 µg/ml of goat anti-MBP antibodies (New England Biolabs) diluted in 100 µl of PBS(-) (phosphate-buffered physiological saline not containing divalent metal ions) was introduced into wells of a microtiter plate and left standing at room temperature for 8 hours or longer to coat the wells with the antibodies. Each well was washed with 200 µl of PBS(-). Then, 1% BSA (bovine serum albumin) dissolved in 200 µl of PBS(-) was added thereto and allowed to react at room temperature for 8 hours or longer to block the wells. Subsequently, each well was washed with 200 µl of PBS(-) containing 0.05% Tween 20. Then, 100 µl of the purified MBP-NS34a solution was added thereto and incubated at room temperature for 2 hours or longer.

Each well was washed with 200 µl of PBS(-) containing 0.05% Tween 20. Then, 100 µl of horseradish peroxidase-conjugated goat anti-MBP antibodies (New England Biolabs) diluted 50,000 times with PBS(-) was added thereto and incubated at room temperature for 2 hours or longer. Each well was washed with 200 µl of PBS(-) containing 0.05% Tween 20. Then, 100 µl of ABTS (2,2'-azino-di-(3-ethyl-benzthiazoline-sulfonic acid)) solution (Amersham Pharmacia Biotech) was added thereto and incubated at room temperature for 5 minutes. 100 µl of 1 M NaOH (Wako Pure Chemical Industries) was added thereto to stop the reaction.

The optical density at 450 nm of the reaction mixture was measured and the MBP-NS34a was quantified as the MBP equivalent based on a calibration curve. The calibration curve was prepared by performing similar ELISA using MBP (New England Biolabs) having known concentrations in a range of 20 to 300 ng/ml.

Example 2: Screening of MBP-NS34a-binding phage by panning method

The panning method was performed basically according to the method of Smith et al. (Methods in Enzymology, 217, 228-257) and the protocol attached to a phage display system manufactured by Pharmacia. Maltose was added at a final concentration of 10 mM to the MBP-NS34a protein and the phage library.

<1> Coating of polystyrene tube with target protein

The protein (MBP-NS34a) solution used for panning and ELISA was prepared at a concentration of 10 µg/µl by using a protein coating buffer (10 mM NaHCO₃ buffer, pH 9.6). A 6-ml polystyrene tube (Falcon 2063) was filled with about 5 ml of the protein solution and coated with the protein on a PALM REACTION PR-12 (NEWCO) overnight at 4°C. The protein solution was removed and the tube was washed once with TBS buffer. Then, the tube was filled with about 5 ml of a blocking solution (10 mM NaHCO₃ buffer containing 5% BSA) and shaken on the PALM REACTION PR-12 at 4°C

for 2 hours. The blocking solution was removed and the tube was washed with TBST (TBS containing 0.5% Tween 20) buffer six times. Then, a predetermined amount of phage library (about 2×10^{12} TU) prepared with TBST buffer containing 1 mg/ml (0.1%) of BSA was added and the tube was shaken on the PALM REACTION PR-12 overnight at 4°C.

<2> Washing and elution of phage

The phage solution was removed from the tube by using a pipette and the tube was washed with TBST buffer 10 times. 4 ml of elution buffer (0.1 N glycine-HCl containing 0.1% BSA, pH 2.2) was filled and the tube was shaken on the PALM REACTION PR-12 for 15 minutes to elute the phages binding to the tube, and the eluate was neutralized with 400 μ l of 2 M Tris solution (pH unadjusted).

<3> Reinfection and rescue of phage and measurement of collected phage

One of colonies of the *Escherichia coli* TG1 strain grown on a minimum medium agar plate was cultured overnight at 37°C in 5 ml of LB medium by using a shaking incubator. 0.2 ml of the TG1 culture solution was cultured with shaking in 20 ml of 2 x YT medium at 37°C for about 3 hours to obtain a culture solution having an OD of about 1.0, and it was left standing at room temperature for 5 minutes. A half

amount of the eluted phage solution collected in the above <2>, i.e., 2.2 ml, and an equal volume of the TG1 culture solution were mixed and shaken at 37°C for 15 minutes. A part of the mixture was streaked on an SOBAG plate and incubated overnight at 37°C. The number of collected phage clones was calculated and templates for sequencing were prepared. Microbial cells were collected from the remaining mixture by centrifugation (4°C, 3000 rpm, 15 minutes), resuspended in 2 x YT-AK medium containing 40 mg/ml of kanamycin and 40 mg/ml of ampicillin and cultured at 37°C overnight with shaking.

Similarly, the culture solution was subjected to precipitation with polyethylene glycol to collect the amplified phages. The culture solution was collected by centrifugation (4°C, 10,000 rpm, 10 minutes), and PEG/NaCl solution was added in a 0.15-fold volume. The mixture was left standing at 4°C for 4 hours or longer. Then, the mixture was centrifuged again (4°C, 10000 rpm, 10 minutes), and the precipitate was suspended in 1 ml of TBS buffer. Finally, centrifugation (4°C, 3000 rpm, 15 minutes) was performed and the supernatant was collected into a 1.5-ml Eppendorf tube so that insoluble matters were not involved. The titer of the phage was measured according to the phage titration method.

The phage clones binding to MBP-NS34a were concentrated by repeating the above operations of <1>

to <3> three to four times. The second and subsequent cycles were performed according to <1> and <2> by using phages (10^{11} - 10^{12} TU/ml) obtained by amplifying the phages collected in each previous screening. Phages presenting peptides showing affinity for MBP-NS34a were included in the concentrated phages, and it was considered that the peptides were encoded by random DNA corresponding to them.

<4> Phage clones potentially binding to MBP-NS34a and preparation of phage DNA

Phage clones and double-stranded DNA were prepared from the *Escherichia coli* TG1 colonies obtained on the SOBAG agar plate used in the above <3>. The obtained colonies on the plate were cultured overnight in 2 x YT-AG medium containing 40 µg/ml ampicillin. 50 µl of the culture solution was transferred into a tube to prepare the phage. Sequencing templates were prepared by using the remaining 950 µl. To 50 µl of the culture solution, the M13K07 helper phage at an m.o.i. of 10 and 500 µl of 2 x YT-AG medium were added, and the mixture was shaken at 37°C for 30 minutes. Microbial cells were collected by centrifugation (4°C, 3000 rpm, 15 minutes), resuspended in 1 ml of 2 x YT-AK medium and cultured overnight at 37°C with shaking. The following day, the culture solution was collected in an Eppendorf tube by centrifugation (4°C, 3000 rpm, 15

minutes). Further, when it was considered that there were only a small number of phage particles, a concentrated phage solution was prepared by using the PEG/NaCl solution in a 0.15-fold amount. These phages were assumed to be phage clones potentially binding to MBP-NS34a and the binding property was determined according to the ELISA method described below. The template DNA for sequencing was prepared by using a FlexiPrep Plasmid Extraction Kit (Amersham Pharmacia Biotech) according to the attached protocol.

<5> Identification of phage that binds to MBP-NS34a by ELISA method

The protein (MBP-NS34a) was immobilized on a 96-well microtiter plate (Maxisorp, Nunc) by using 50 μ l of the solution per well in the same manner as in the aforementioned panning method (overnight at 4°C, 10 mM NaHCO₃ buffer, pH 9.6, protein concentration of 10 μ g/ml). The plate was blocked by introducing 200 μ l of 5 mg/ml (0.5%) BSA fraction V solution prepared by 10 mM NaHCO₃ per well and leaving it at 4°C for 1 hour as in the panning method. After rinsed with TBS buffer once, 50 μ l of the phage solution prepared in <4> was added to each MBP-NS34a-immobilized well, and incubated overnight at 4°C. Each well was washed with TBS buffer four times, and 50 μ l of horseradish peroxidase (HRP)-conjugated sheep anti-M13 phage antibodies (Amersham Pharmacia Biotech, Code No.27-

9411-01) diluted 2000 times by using 5% skim milk was added per well and incubated at room temperature for 1 hour.

Each well was washed with 200 μ l of TBS containing 0.5% Tween 20 five times. Then, 100 μ l of color-developing substrate solution (50 mM citric acid containing 0.2 mg/ml ABTS, pH 4.0; Amersham Pharmacia Biotech, Code No. 27-9402-01) was added and further incubated at room temperature. The optical density was measured at 405 nm by a 96-well plate reader (BIO-RAD). When the optical density of MBP-NS34a was at least twice as high as the optical density obtained when MBP was used instead of MBP-NS34a, the solution was determined to contain a phage clone binding to MBP-NS34a. The affinity for the NS3 protease, of the phages having peptides selected according to the present invention was determined by the ELISA method and the results are shown in Fig. 1. As the negative control, the M13 phage that expressed no random peptide was used.

<6> Analysis of nucleotide sequences of phage clones that bind to MBP-NS34a

Plasmid was prepared from 950 μ l of culture solution by using a FlexiPrep kit (Amersham Pharmacia Biotech). 2.5 μ l (about 100 ng) of DNA corresponding to the phage identified as described above among the phagemid DNA obtained in <4> and 1.6 pmol of

sequencing primer 5'-TGAATTTTCTGTATGGGG-3' (SEQ ID NO: 18) prepared so that it should bind to a position about 60 nucleotide upstream from the random DNA region on the phage were mixed. A cycle sequencing reaction was performed by using a Prism DNA Cycle Sequencing Kit (PE Biosystems) and a PCR apparatus, PCR 9600 (Perkin-Elmer), according to the attached protocol. After the completion of the reaction, the reaction product was collected by ethanol precipitation and dissolved in formamide. Each DNA sequence was determined by using a model 377 DNA sequencer (ABI) and the amino acid sequence in the random region of the phage was analyzed. The amino acid sequences and appearance frequency are shown in Table 1.

Table 1

| Clone | Amino acid sequence | | Frequency |
|-------|---------------------|-----------------|-----------|
| K13 | CVPLVCIFRC | (SEQ ID NO: 1) | 19 |
| J94 | CSRIVCLLWC | (SEQ ID NO: 2) | 5 |
| J93 | CWLFLWC | (SEQ ID NO: 3) | 3 |
| N59 | CWLLVFC | (SEQ ID NO: 4) | 2 |
| K5 | CIAVIC | (SEQ ID NO: 5) | 2 |
| K25 | CRPVMALFYC | (SEQ ID NO: 6) | 2 |
| N28 | IWAVLWIWN | (SEQ ID NO: 7) | 2 |
| J95 | WVFFWLSRP | (SEQ ID NO: 8) | 1 |
| K1 | IWHFSFMWI | (SEQ ID NO: 9) | 1 |
| N50 | CRLLVKVFWC | (SEQ ID NO: 10) | 1 |
| N51 | GRRFGIVCTCLKYFV | (SEQ ID NO: 11) | 1 |
| N70 | CALMSCLFWC | (SEQ ID NO: 12) | 1 |

<7> Preparation of synthetic peptides

The sequences of the peptides obtained from the phage peptide library are shown in Table 1. These

peptides can have either a straight-chain structure or an intramolecular cyclic structure, in which two cysteine residues form a disulfide bond. Chemical synthesis of the peptides was entrusted to Sawady Technology. Various peptides having a straight-chain or cyclic structure were synthesized by the solid phase method and purified to 80% purity or higher by reverse phase HPLC.

(Conditions of liquid chromatography)

Column: Kromasil 100 C18, 5 μ m, 125 x 4 mm

Temperature: 20°C

Flow rate: 0.75 ml/min

Eluant: Buffer A = 0.1% trifluoroacetate aqueous solution

Solvent: 0.1% TFA (trifluoroacetate) aqueous solution/acetonitrile (20/80)

All the synthesized peptides were confirmed for their molecular weights by mass spectroscopy and used for evaluation. Sequences of the synthesized peptides are shown in Table 2.

Example 3: Examination of protease inhibitory ability of synthetic peptides

<1> NS3 protease inhibitory activity test using substrate cleavage reaction as index

An NS3 protease cleavage sequence between NS5a and NS5b in the HCV polypeptide was synthesized. The

protease inhibitory ability of each of the synthetic peptides was examined according to the method of Kakiuchi et al., in which a synthetic substrate having an amino terminus labeled with a dansyl group was reacted in the presence of a protease and a cleavage degree is measured by reverse phase HPLC (Biochem. Biophys. Res. Commun., 210, 1059, 1995). 1 μ g of the purified MBP-NS34a and a reaction mixture (50 mM Tris-HCl (pH 7.5), 30 mM NaCl, 5 mM MgCl₂ and 10 mM DTT) containing an evaluation sample prepared at an appropriate concentration were incubated at 25°C for 15 minutes. To the solution, the synthesized substrate was added at a final concentration of 50 μ M, and the mixture was allowed to react at 37°C for 1 to 3 hours. Subsequently, the enzymatic reaction was stopped by adding a reaction-stopping solution (20% acetonitrile, 0.1% TFA) in a volume 9 times as much as the reaction volume. Then, the cleaved peak of the synthesized substrate was detected (excited at 340 nm and measured at 510 nm) by a reverse phase column (YMC-AM-302) to calculate the cleavage rate when the inhibitor was added. Further, a value obtained by dividing this cleavage rate when the inhibitor was added by the cleavage rate when no inhibitor was added, was subtracted from 1 and then multiplied by 100. The obtained value was used as the inhibition rate (%) of the evaluated drug. In the above method, the peptide concentration inhibiting 50% of the protease activity

(IC₅₀) was calculated (Table 2).

Table 2

| Clone | Amino acid sequence | | IC ₅₀ (μM) |
|-------|---------------------|-----------------|-----------------------|
| K13 | CVPLVCIFRC | (SEQ ID NO: 1) | 5 |
| J94 | CSRIVCLLWC | (SEQ ID NO: 2) | 5 |
| J93 | CWLFLWC | (SEQ ID NO: 3) | 3 |
| N59 | CWLLVFC | (SEQ ID NO: 4) | 3 |
| J95 | WVFFWLSRP | (SEQ ID NO: 8) | 15 |
| K1 | IWHFSFMWI | (SEQ ID NO: 9) | 15 |
| N51 | GRRFGIVCTCLKYFV | (SEQ ID NO: 11) | 7 |

As a result, it was revealed that the above synthetic peptides had an activity for inhibiting the NS3 protease activity.

<2> Inhibition of binding of phage peptide and MBP-NS34a by NS4a peptide, 4A18-40

A possibility that an action site of each phage peptide clone obtained as described above and that of the NS4a peptide derived from virus known to be activated by the binding to the NS3 protein were identical or overlapped was examined. A partial peptide of the NS4a peptide composed of the 18th to 40th residues from the amino terminus (referred to as 4A18-40 peptide) is known as a minimal region required to enhance the NS3 protease activity (Tanji et al., J. Virol., 69, 4017-4026, 1995). Accordingly, an influence on the binding of each phage peptide and the MBP-NS34a protein was analyzed by the phage ELISA method using a chemically synthesized 4A18-40 peptide

(LTTGSSVVIVGRIILSGRPAVVDP, SEQ ID NO: 19). 10 µg/ml of the MBP-NS34a protein to which the 4A18-40 peptide was added in an amount of 10 or 20 times in excess in a molar ratio was immobilized on each well of a 96-well microtiter plate. The plate was blocked by using 100 µl of 0.5% BSA solution prepared with 10 mM NaHCO₃, and rinsed once with 200 µl of TBS buffer. 100 µl of the phage solution prepared in Example 2, <4> was added to each well of the 96-well plate on which the NS4a peptide and the MBP-NS34a protein were immobilized, and incubated overnight at 4°C. Each well was washed with 200 µl of TBS buffer four times. Then, 50 µl of horseradish peroxidase (HRP)-conjugated sheep anti-M13 phage antibodies diluted 2000 times with 5% skim milk was introduced into each well and incubated at room temperature for 1 hour.

Each well was washed with 200 µl of TBS containing 0.5% Tween 20 five times. Then, 100 µl of a peroxidase color-developing substrate solution was added thereto and further incubated at room temperature. The optical density at 405 nm was measured by a 96-well plate reader (BIO-RAD). The optical density due to binding of the MBP-NS34a and the phage peptide observed in the presence of the NS4a peptide was measured. As a result, binding of the phage clone K13 to the MBP-NS34a protein was increasingly inhibited as the abundance ratio of the 4A18-40 peptide rose to 10-fold and 20-fold (Fig. 2).

<3> Screening of inhibitor for binding of phage peptide and MBP-NS34a

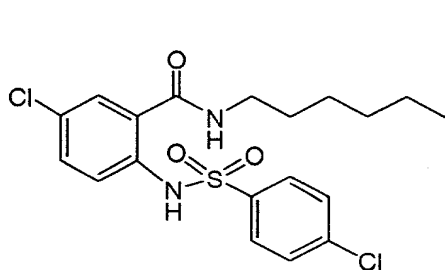
A binding inhibitor was screened by using the above phage ELISA system. As peptides used for screening, the phage peptides whose protease inhibitory activity was confirmed by using the synthetic peptides were prepared in an amount required for screening according to the method of Example 2 <3>. Each well of a 96-well microtiter plate was coated by using 50 μ l of the MBP-NS34a protein solution prepared at 10 μ g/ml. The plate was blocked by using 100 μ l of 0.5% BSA solution prepared with 10 mM NaHCO₃ and rinsed once with 200 μ l of TBS buffer for each well. A mixed solution of a compound in a chemical library (synthesized in our company) diluted to an appropriate concentration and each phage prepared as described above was prepared, and the mixture was added to each well coated with the MBP-NS34a protein in an amount of 100 μ l and incubated overnight at 4°C. Each well was washed with 200 μ l of TBS buffer four times. Then, 50 μ l of horseradish peroxidase (HRP)-conjugated sheep anti-M13 phage diluted 2000 times with 5% skim milk was introduced into each well and incubated at room temperature for 1 hour.

Each well was washed with 200 μ l of TBS containing 0.5% Tween-20, five times. Then, 100 μ l of ABTS solution was added thereto and further incubated

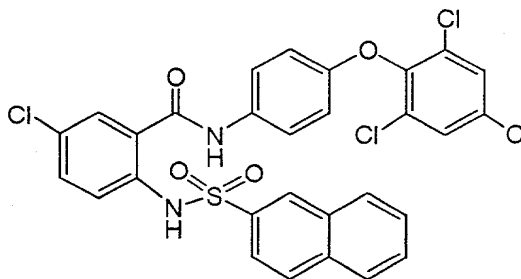
at room temperature. The optical density at 405 nm was measured by using a 96-well plate reader (BIO-RAD). There was selected Compound HCP1271 (Compound 5 described in Japanese Patent Laid-Open (KOKAI) No. 2000-7645), in the presence of which the optical density by binding of the MBP-NS34a and the phage peptide was lower than the optical density observed in the absence of the compound. Further, this inhibitory compound was serially diluted and its influence on the binding of 5 kinds of other phage peptides, which inhibited the NS3 protease activity, was similarly determined by the phage ELISA method. As a result, HCP1271 concentration-dependently inhibited the binding of the MBP-NS34a and the phage peptide (Fig. 3). As a result of measurement of the protease activity inhibitory ability of this compound according to the method of Example 3, <1>, it was found that HCP1271 strongly inhibited the NS3 protease activity with an IC_{50} value of 2.2 μM . Further, since a compound HCP1231 similar to HCP1271, was retrieved by the search of the chemical library, its NS3 protease activity inhibitory ability and influence on the binding of the phage peptide and the NS3 protease were examined. As a result, it showed very weak protease activity inhibitory activity and scarcely inhibited the binding of the phage peptide and the NS34a (Fig. 4). That is, it was shown that the compound obtained from screening of the inhibitor for binding of the

phage peptide and the protease protein could inhibit the protease activity very well.

The structural formulae of the compounds HCP1231 and HCP1271 are shown below.



HCP1231



HCP1271

Industrial Applicability

The method of the present invention is useful as a simple method for screening drugs inhibiting an interaction between various biological components such as an interaction between proteins or between protein and nucleic acid.

The peptide compound of the present invention has affinity for the HCV serine protease and is useful as an active ingredient of a preventive and/or therapeutic drug for diseases induced by abnormality of liver cells due to infection and proliferation of hepatitis C virus, for example, cirrhosis and liver cancer. Moreover, the screening method of the present invention is useful as a method for selecting a compound capable of modulating a protein function in search of therapeutic drugs for diseases.

WHAT IS CLAIMED IS:

1. A method for screening a substance which interacts with a specific region of a biomolecule having an activity, to regulate the activity, said method comprising the following steps:
 - (a) a step of selecting a recombinant organism that interacts with the biomolecule, from a peptide library composed of a collection of recombinant organisms each presenting at least one of various peptides on its surface, with a proviso that the interaction is not an antigen-antibody reaction; and
 - (b) a step of selecting a substance inhibiting the interaction between the selected recombinant organism or a peptide presented by the recombinant organism and the biomolecule.
2. The method according to Claim 1, wherein the biomolecule is a protein, a nucleic acid or a sugar chain.
3. The method according to Claim 1, wherein the peptide library is composed of random peptide-presenting phage.
4. The method according to Claim 1, wherein the peptide library is composed of random peptide-presenting *Escherichia coli*.

5. The method according to Claim 1, wherein the peptides each have a length of 3 to 15 residues.

6. The method according to Claim 1, wherein the selected recombinant organism or the peptide presented by the recombinant organism is labeled with a labeling substance.

ABSTRACT OF THE DISCLOSURE

A method for screening a low molecular weight substance which binds to a specific site of a biomolecule and may be usable as a starting material in drug development. This method comprises screening a substance that interacts with a specific region of a biomolecule having an activity, to regulate the activity, by the following steps:

- (a) a step of selecting a recombinant organism that interacts with the biomolecule, from a peptide library composed of a collection of recombinant organisms each presenting at least one of various peptides on its surface; and
- (b) a step of selecting a substance inhibiting the interaction between the selected recombinant organism or a peptide presented by the recombinant organism and the biomolecule.

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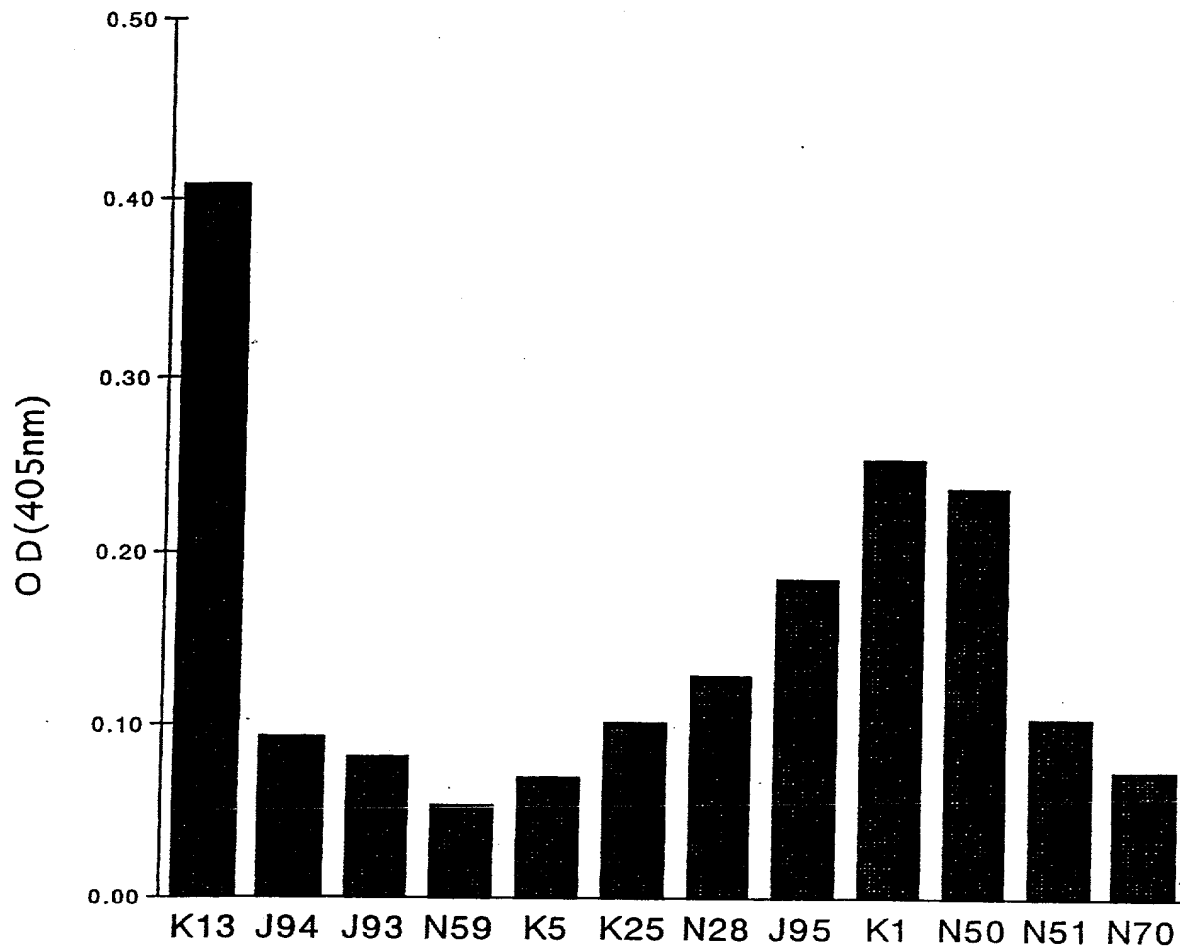


Fig. 1

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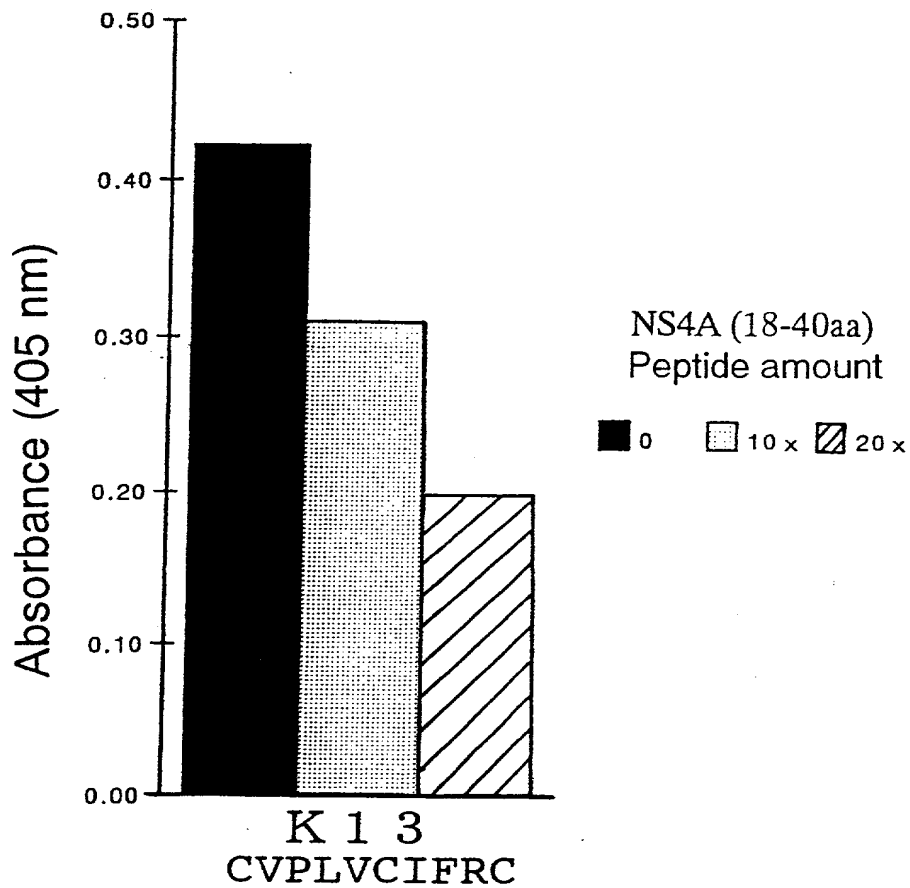


Fig. 2

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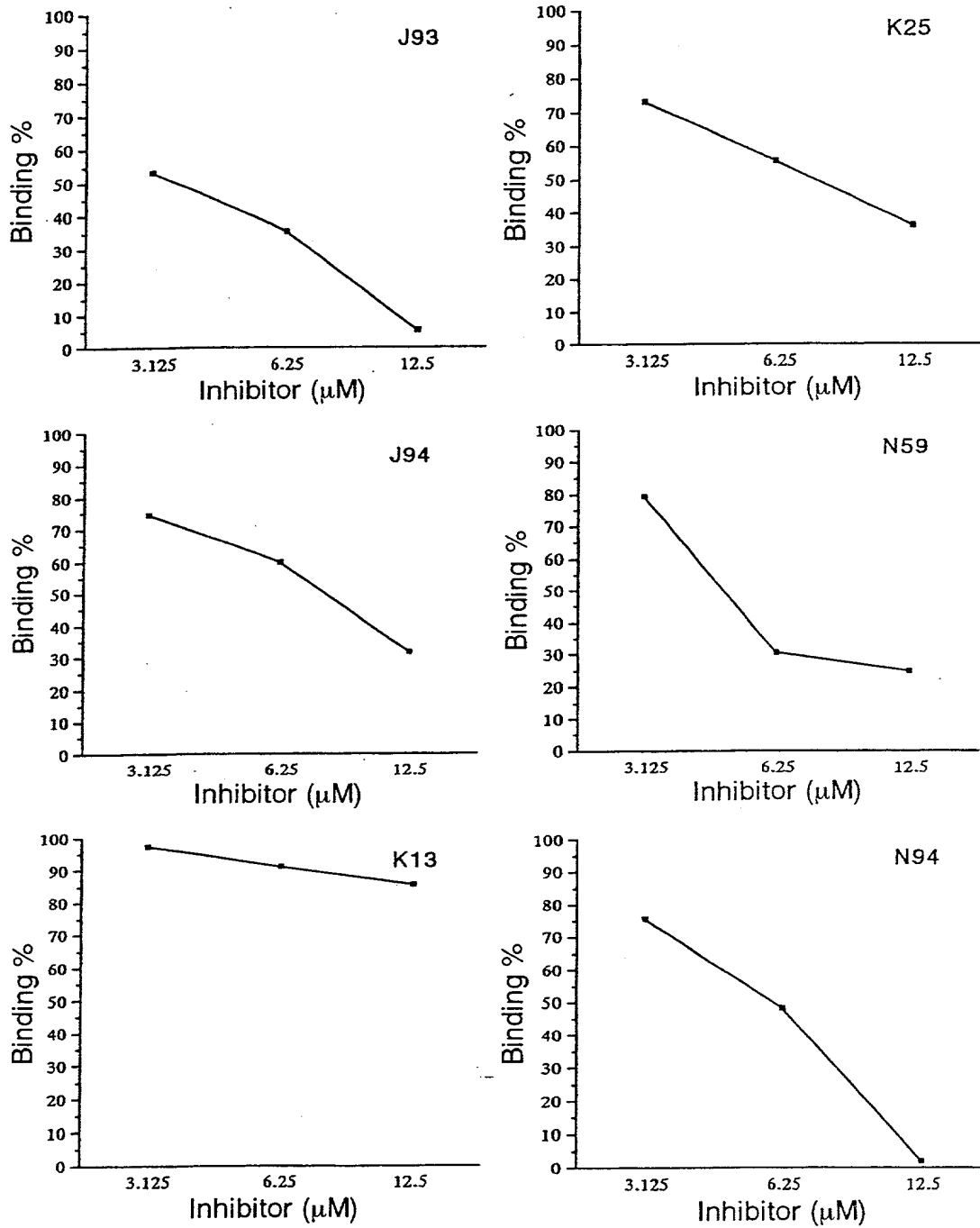


Fig. 3

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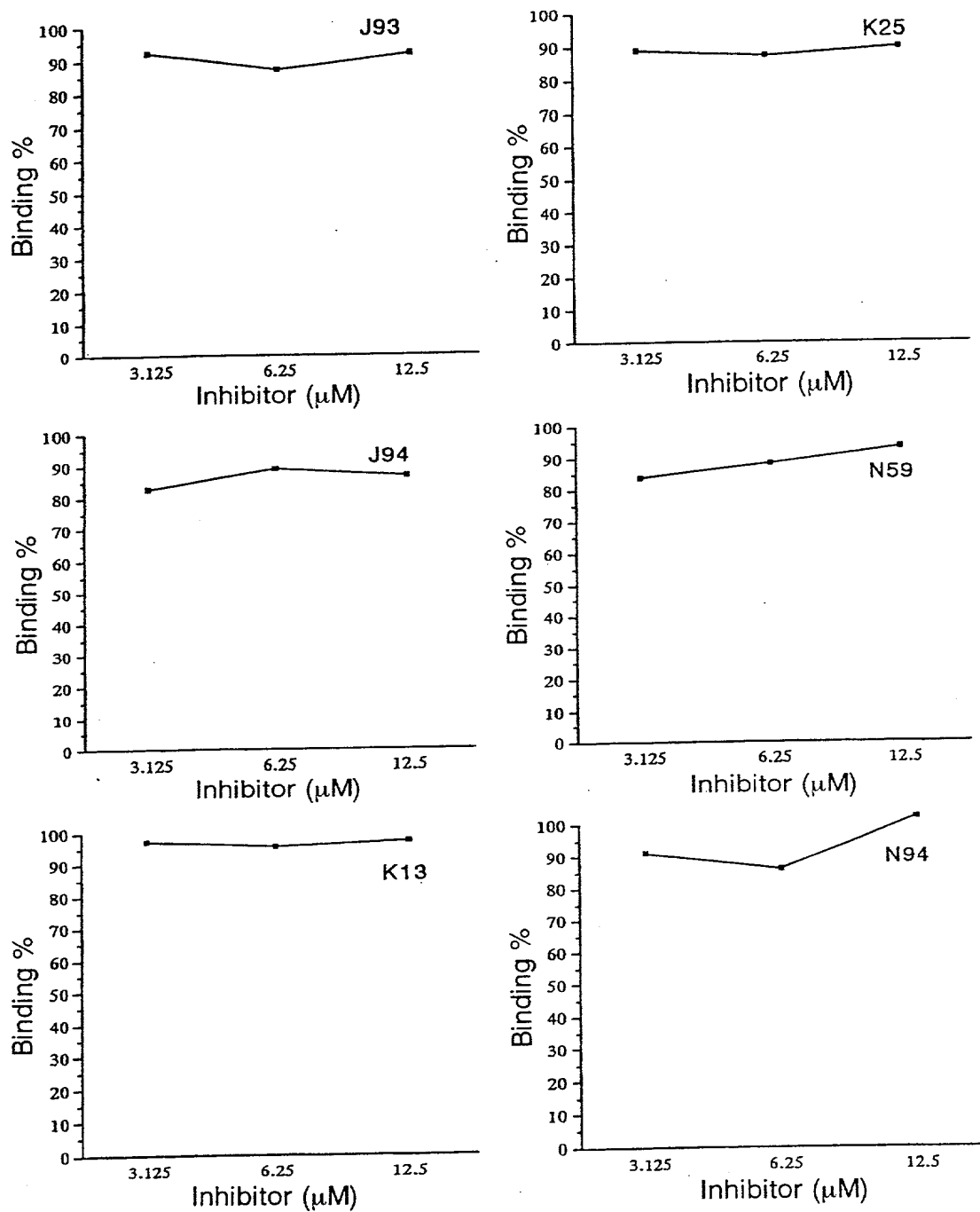


Fig. 4

Declaration, Power Of Attorney and Petition

Page 1 of 3

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Method for Screening Biomolecule Activity Regulator

the specification of which

☒ is attached hereto.

☐ was filed on _____ as

Application Serial No. _____

and amended on _____

☒ was filed as PCT international application

Number PCT/JP 00/01478 /

on March 10, 2000 /

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

| Application No. | Country | Day/Month/Year | Priority Claimed |
|-------------------|----------------|---------------------|---|
| <u>11-63110 /</u> | <u>Japan /</u> | <u>10/03/1999 /</u> | <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No |
| _____ | _____ | _____ | <input type="checkbox"/> Yes <input type="checkbox"/> No |
| _____ | _____ | _____ | <input type="checkbox"/> Yes <input type="checkbox"/> No |
| _____ | _____ | _____ | <input type="checkbox"/> Yes <input type="checkbox"/> No |

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

| | |
|----------------------|---------------|
| _____ | _____ |
| (Application Number) | (Filing Date) |
| _____ | _____ |
| (Application Number) | (Filing Date) |

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

| Application Serial No. | Filing Date | Status (pending, patented, abandoned) |
|------------------------|-------------|---------------------------------------|
| _____ | _____ | _____ |
| _____ | _____ | _____ |
| _____ | _____ | _____ |

And we (I) hereby appoint: Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Robert F. Gnuse, Registration Number 27,295; Jean-Paul Lavalleye, Registration Number 31,451; Timothy R. Schwartz, Registration Number 32,171; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,893; Richard L. Treanor, Registration Number 36,379; Steven P. Weihrouch, Registration Number 32,829; John T. Goolkasian, Registration Number 26,142; Marc R. Labgold, Registration Number 34,651; William J. Healey, Registration Number 36,160; and Richard L. Chinn, Registration Number 34,305; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, MCCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Signature of Inventor

Date

NAME OF FIFTH JOINT INVENTOR

Signature of Inventor

Date